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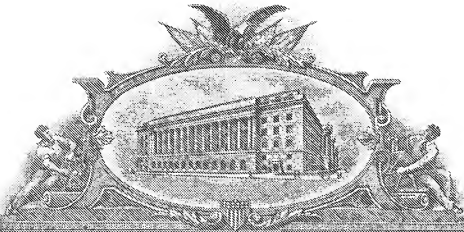Document type: Certified copy of priority document

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Applicant(s):   CHARLES L. SAWYERS, CHARLIE D. CHEN, and DEREK S. WELSBIE

Docket: G&C 30435.157-US-P1

Title: METHODS AND MATERIALS FOR ASSESSING PROSTATE CANCER THERAPIES

MAIL STOP PROVISIONAL PATENT APPLICATION
Commissioner for Patents
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Dear Sir:

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. 1.53(c) on behalf of the
following inventors:

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Enclosed herewith are the following application parts:

☒ Transmittal sheet, in duplicate, containing Certificate Of Mailing Under 37 CFR 1.10.
☒ Provisional Patent Application: 33 page(s) of a specification, 1 page(s) of an Abstract.
☒ 12 sheets of drawings.
☒ A charge to Deposit Account in the amount of $80.00 to cover the Filing Fee.
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METHODS AND MATERIALS FOR ASSESSING PROSTATE CANCER THERAPIES

Statement of Government Support

Field of the Invention

The present invention relates to methods and materials for assessing prostate cancer therapies.

Background of the Invention

Cancer is the second leading cause of human death next to coronary disease. Worldwide, millions of people die from cancer every year. In the United States alone, as reported by the American Cancer Society, cancer causes the death of well over a half-million people annually, with over 1.2 million new cases diagnosed per year. While deaths from heart disease have been declining significantly, those resulting from cancer generally are on the rise. In this century, cancer is predicted to become the leading cause of death.

Worldwide, several cancers stand out as the leading killers. In particular, carcinomas of the lung, prostate, breast, colon, pancreas, and ovary represent the primary causes of cancer death. These and virtually all other carcinomas share a common lethal feature. With very few exceptions, metastatic disease from a carcinoma is fatal. Moreover, even for those cancer patients who initially survive their primary cancers, common experience has shown that their lives are dramatically altered.

Adenocarcinoma of the prostate is the most frequently diagnosed cancer in men in the United States, and is the second leading cause of male cancer deaths (Karp et al., Cancer Res. 56:5547-5556 (1996)). Therapy for prostate cancer is typically initiated using hormone drugs that lower serum testosterone, often given in combination with competitive androgen receptor (AR) antagonists. Although initially effective at blocking tumor growth, these therapies eventually fail, leading to a drug resistant stage called androgen independent or hormone refractory (HR) disease that is uniformly lethal.

Postulated mechanisms to explain resistance to hormone therapy can be

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separated into three general categories[1-3]. The first includes DNA-based alterations in the *AR* gene such as amplification or point mutations, which collectively only occur in a minority of patients[4-7]. A subset of these *AR* mutations map to the ligand binding domain (LBD) and are proposed to cause resistance by altering the response of the receptor such that noncanonical ligands like estrogen or hydrocortisone, or even AR antagonists like flutamide, behave as agonists[8,9]. Although their clinical association with antiandrogen resistance is strong, the overall frequency of *AR* amplification or mutation cannot account for most cases of hormone refractory disease.

The second category applies to the majority of patients without *AR* gene mutation or amplification who retain active AR signaling. Increased mitogen-activated protein kinase signaling mediated by oncogenes such as ErbB2 or Ras can cause ligand-independent activation of AR[10,11]. The kinases and substrates responsible for AR activation in this setting are unknown, but this is presumed to occur through downstream phosphorylation of AR-associated proteins or AR itself, analogous to the estrogen receptor (ER)[12-14]. Similarly, alteration in the balance of coactivators or corepressors can affect AR activation[15,16], based on similar findings for ER[17]. The relative frequency of these events and their relationship to clinical drug resistance remain to be defined.

The third category of hormone resistance mechanisms is based on the concept that the pro-growth and survival functions of AR can be "bypassed" by alternative signaling pathways, such that AR is no longer relevant to disease progression. One example is upregulation of the anti-apoptotic gene Bcl-2 in late stage clinical samples[18,19], but functional proof of a role in hormone resistance is lacking. The AR bypass hypothesis is also consistent with observations of *AR* gene methylation leading to decreased or absent AR expression in some HR cancers[20], as well as reports that androgen induces growth arrest or apoptosis in certain contexts[21,22].

Collectively, these data implicate multiple mechanisms by which prostate cancers acquire resistance to hormone therapy and highlight the continuing debate about the role of AR in late stage disease progression. Consequently, there is a need in the art for models that reproduce clinically significant aspects of this disease progression, particularly the transition from the initial stage in the prostate cancer where the cancer cells are sensitive to hormone antagonists to the subsequent drug resistant stage. In

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particular, a well-defined and manipulatable cell based model is needed to dissect the molecular events associated with the progression from a drug sensitive to a drug resistant phase. In addition, there is a need in the art for cell based prostate cancer models that reproduce the drug sensitive and/or drug resistant phases of cancers of the prostate that

5   can be used, for example in the evaluation of new therapeutic modalities. The invention disclosed herein satisfies this need.

<div align="center">

Summary of the Invention

</div>

Using microarray-based profiling of isogenic prostate cancer xenograft models,

10   we found that a modest (3-5 fold) increase in androgen receptor (AR) mRNA was the only expression change consistently associated with developing resistance to antiandrogen therapy. This increase in AR mRNA and protein was both necessary and sufficient to convert prostate cancer growth from a hormone sensitive to hormone refractory stage, and was dependent on a functional ligand-binding domain.

15   Furthermore, AR antagonists displayed agonist activity in cells with increased AR levels, and this antagonist/agonist conversion was associated with alterations in the pattern of coactivators and corepressors recruited to the promoter of AR target genes. Increased levels of AR confer resistance to anti-androgens by amplifying signal output from low levels of residual ligand and altering the normal response to antagonists. The disclosure

20   provided herein that is based upon these findings includes assays for examining the effects of therapeutic compounds on mammalian cells such as androgen independent prostate cancer cells and further provides insight toward the design of novel antiandrogens.

One embodiment of the invention is a method of testing compounds for an

25   effect on a mammalian prostate cancer cell comprising contacting the compound to be tested with a the mammalian prostate cancer cell wherein the mammalian cancer cell expresses an exogenous wild type androgen receptor (AR) polynucleotide that encodes the AR polypeptide of SEQ ID NO:1 such that the levels of mRNA in the cell that encode the AR polypeptide or the AR polypeptide are at least about 2 fold higher than

30   normal/endogenous AR mRNA levels in a mammalian prostate cell; and then comparing one or more characteristics of the mammalian prostate cancer cell to which the compound was administered with the same one or more characteristics of a control

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mammalian prostate cancer cell to which the compound has not been administered, wherein a difference in one or more of the one or more characteristics indicates that the compound has an effect on the mammalian prostate cancer cell.

Another embodiment of the invention is a method of examining the physiological effect of a compound on a mammalian prostate cancer cell, the method comprising contacting the compound to be tested with a mammalian prostate cancer cell, wherein the mammalian prostate cancer cell expresses either an exogenous wild type polynucleotide that encodes the AR polypeptide of SEQ ID NO:1, or a polynucleotide that encodes the AR polypeptide of SEQ ID NO:1 having a deletion, insertion or substitution of at least one amino acid in the AR polypeptide of SEQ ID NO:1, such that the total levels of mRNA in the cell that encode the AR polypeptide or the total levels of AR polypeptide are at least 2 fold higher than normal/endogenous AR mRNA or AR polypeptide in the cell; and then examining one or more physiological characteristics of the mammalian prostate cancer cell to which the compound is administered, so that the physiological effect of the compound on the mammalian prostate cancer cell is examined. A related embodiment of the invention is a method further comprising examining the physiological effect of a plurality of compounds on the mammalian prostate cancer cell, wherein an observable difference in one or more physiological characteristics exerted by a first compound as compared to one or more physiological characteristics exerted by a second compound indicates that the first compound has a stronger or weaker physiological effect than the second compound on the mammalian prostate cancer cell. Typically the method is performed in a high throughput format. Alternatively, the method is performed in a low throughput format. Compounds tested in such assays are typically antagonists or agonists. In practice we define a drug as an AR antagonist when the drug inhibits or competes for the binding of a ligand or a stimulus and inhibits the biological function of the androgen receptor. A drug is defined as an AR agonist when the drug stimulates or activates the biological function of the androgen receptor.

Yet another embodiment of the invention is a method of testing one or more compounds for an effect on a mammalian cell, the method comprising contacting at least one compound to be tested with the mammalian cell, wherein the mammalian cell expresses an exogenous wild type or mutated protein of interest, such as the estrogen

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receptor, such that the total levels of mRNA in the cell that encode the protein of interest or the total protein levels of the protein of interest are at least 2 fold higher than normal/endogenous mRNA or polypeptide of the protein of interest, such as the estrogen receptor, in the cell, comparing one or more characteristics of the mammalian cell to which the compound is administered with the same one or more characteristics of a control mammalian cell to which the compound has not been administered, wherein a difference in one or more characteristics indicates that the compound has an effect on the mammalian cancer cell or mammalian cell. In such methods, the mammalian cell is typically a cancer cell, for example a breast, ovarian or prostate cancer cell. A related embodiment of the invention is a method further comprising examining the physiological effect of a plurality of compounds on the mammalian cell, wherein an observable difference in one or more physiological characteristics exerted by a first compound as compared to one or more physiological characteristics exerted by a second compound indicates that the first compound has a stronger or weaker physiological effect than the second compound on the mammalian cell.

Another embodiment of the invention is a method of treating a hormone refractory prostate cancer in a patient, the method comprising administering to the patient an agent that decreases or affects the biological function of the androgen receptor by affecting the androgen receptor ligand-binding, nuclear translocation, or by affecting DNA-binding, or through altering formation of coactivator or corepressor complexes associated with the androgen receptor.

Another embodiment of the invention is a method of treating a hormone refractory prostate cancer in a patient, the method comprising administering to the patient and an agent that decreases or affects the biological function of the androgen receptor through affecting the androgen receptor DNA levels, androgen mRNA levels, or androgen protein levels. In such methods, the androgen receptor protein level can be decreased through modulation of signal transduction pathways such as targeting EGF receptors that crosstalk to the androgen receptor. Alternatively, the androgen receptor protein level is decreased by the induction of cellular degradation pathways such as proteosome degradation machinery. Alternatively, the androgen receptor protein level is decreased by dissociating the androgen receptor from heat shock proteins that maintain the androgen receptor integrity. Preferably the androgen receptor protein level is

decreased using androgen receptor antisense or mRNA knockdown technology. We consider that any one of these above manipulations or combination of any of these manipulations would affect the biological function of the androgen receptor. The preferred way of these methods would be to use an agent to disrupt or reduce the ligand binding of the androgen receptor

Another embodiment of the invention is a method of treating a hormone refractory prostate cancer in a patient, the method comprising administering to the patient and an agent that decreases or affects the biological function of the androgen receptor through modifying the androgen receptor protein. Optionally, the androgen receptor protein is modified by modifying the polynucleotide or polypeptide sequence of the androgen receptor or by posttranslational modifications of the androgen receptor including but not restricted to phosphorylation, acetylation, ubiquitination, and sumolation.

Another embodiment of the invention is a method of treating a disease or condition, which is resistant to a drug, or a treatment, or combination of a drug and a treatment by increasing the concentration of the protein that is the target of the drug or treatment, the method comprising administering one or more agents or utilizing a technique that affects the biological function of the protein through means described in the paragraphs above. Typically the disease or condition is hormone refractory prostate cancer, in which the androgen receptor DNA, mRNA, or protein levels is increased in prostate cancer cells after surgical or medical castration, or treatments with anti-androgen therapy, or the combination of castration and anti-androgen therapy. In an illustrative embodiment, the disease or condition is hormone refractory breast cancer, in which the estrogen receptor DNA, mRNA, or protein levels is increased in breast cancer cells after hormone therapy such as treatments with tamoxifen or raloxifene.

In a further embodiment of the invention, there are provided articles of manufacture and kits containing materials useful for examining compounds such as AR agonists or antagonists using the methods disclosed herein. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The label on the container may indicate directions for either *in vivo* or *in vitro* use, such as those described above. The kit of the invention comprises the container described

above and a second container comprising a buffer. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

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<u>Brief Description of the Figures</u>

Figure 1 – <u>AR expression in HS and HR xenografts</u>   Top, normalized microarray values for *AR* probe sets one and two (Affymetrix IDs 1577 and 1578, respectively) are shown for pools of tumors from each of the fourteen xenografts. Bottom, AR Western blot from one tumor of each xenografts' pool lysed in 2% SDS. AR protein expression in HS LUCaP35 was evident upon longer exposures.

Figure 2 – <u>AR overexpression is sufficient to cause HR progression</u>   (**a**) Western blot of vector-infected and AR-infected LNCaP. (**b** and **c**) HS LNCaP stably overexpressing Flag-tagged, wildtype, human AR or a vector control were androgen-starved by growth in charcoal-stripped serum for 4-5 d. 5×10^4 cells were plated per well in media containing 10% charcoal-stripped serum supplemented with either 0, 20 or 100 pM R1881 (**b**) or in media containing 10% full serum and either 0 or 10 μM bicalutamide (**c**). After 2 weeks, colonies were visualized with crystal violet staining. (**d**) 1×10^6 LAPC4 vector-infected cells (grey) or AR-infected cells (red) were grown subcutaneously in the flank of SCID mice which were either intact (circles, $n = 8$) or castrated (triangles, $n = 12$). Tumor incidence, defined as a tumor reaching 40 mm³, was followed over time. Inset, western blot of vector-infected and AR-infected LAPC4. (**e**) 2×10^6 LNCaP vector-infected cells (grey) or AR-infected cells (red) were grown subcutaneously in the flank of SCID mice which were either intact (circles, $n = 8$) or castrated (triangles, $n = 12$). Tumor incidence, defined as a tumor reaching 40 mm³, was followed over time.

Figure 3 – <u>AR expression is necessary for HS-to-HR progression</u>   (**a**) Schematic of the lentivirus which expresses GFP and a shRNA directed against *AR*. (**b**) 2×10^5 HR LAPC4 cells infected with either the shRNA-expressing (blue, $n = 8$) or control virus (grey, $n = 8$) were implanted subcutaneously into the flanks of castrated male mice and tumor volume measured weekly (* signifies $P < 0.05$, *t*-test). Right inset, western blot of shRNA-expressing and control (empty vector) cells. Left inset, two tumors from each group, approximately 500 mm³ were explanted and analyzed by flow cytometry for

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the percentage of GFP-positive cells (M1 gate). Shown is a representative sample from each (control virus, top; AR shRNA virus, bottom). **(c)** 5×10^4 HR LNCaP cells infected with either the shRNA-expressing (blue, $n = 8$) or control virus (grey, $n = 8$) were implanted subcutaneously into the flanks of castrated male mice and tumor volume (± SEM) measured weekly. Inset, western blot of shRNA-expressing and control (empty vector) cells.

Figure 4 – AR promotes HR progression through a ligand-dependent, genotropic mechanism **(a)** Mutant or wildtype *AR* constructs were transfected into AR-null cells (COS7) and androgen-starved for 48 h. Cells were then incubated with increasing amounts of ^{3}H-R1881 in the presence or absence of 100-fold excess of cold R1881 and bound ligand was measured by scintillation counting. **(b and e)** LNCaP cells expressing the various AR LBD **(b)** or genotropic **(e)** mutants were assayed for HR growth *in vitro* using either the low-androgen sensitization (light blue) or antagonist resistance (crimson) assay as previously described in **2a**. For each, the *y*-axis is the ratio of the number of colonies of the mutant to the vector control at day 14. All experiments were done in duplicate with a representative example shown. Inset, western blot of LNCaP cells stably expressing the mutant *AR* constructs. **(c)** 1×10^6 LNCaP cells overexpressing the various *AR* cDNAs ($n = 10$) or a vector control ($n = 10$) were implanted into the flanks of castrated male SCID mice and tumor volume (± SEM) was measured over time. **(d)** Confocal immunofluorescence performed with a FLAG-specific antibody on LNCaP cells stably expressing FLAG-tagged, triple point mutant, K618, 632, 633M (ΔNLS) or wildtype AR.

Figure 5 – Increased AR expression converts antagonists to agonists **(a and c)** LAPC4 cells overexpressing AR or a GFP control were androgen-starved for 5 d with charcoal-stripped serum and then challenged with bicalutamide **(a)** or cyproterone acetate **(c)** for 96 h. *PSA* and *β-actin* message was assayed using semiquantitative RT-PCR. **(b and e)** Secreted PSA after 48 hour challenge with bicalutamide **(b)** or 17β-estradiol **(e)** was measured via ELISA. **(d)** LNCaP cells stably infected with an AR-expressing or control virus (Neo) were androgen-starved for 5 d and then challenged for 48 h with bicalutamide. *PSA* and *β-actin* were measured as in **b**. **(f)** LNCaP cells stably infected with the AR-expressing virus or the vector control were starved for 5 d and then challenged with either vehicle (V), 10 μM bicalutamide (B), 100 pM R1881 (R) or 1

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nM DHT (D). After 1 h, cells were harvested and processed for chromatin immunoprecipitation[21].

Figure 6 – <u>Increased AR expression globally converts antagonists to weak agonists</u> (a) Diagram of microarray experiment. (b) Duplicates for bicalutamide or vehicle treatment were averaged and queried for the number of probe sets induced 2-fold, $P < 0.05$ with a minimum expression of 500 in at least one sample. (c) Change in expression of the 48 probe sets in AR-overexpressing (aqua) or control cells (red) in response to bicalutamide. (d) Change in expression of the 48 probe sets in response to increasing doses of R1881. (e) Dataset was analyzed for the number of probe sets induced 2-fold with a minimum expression of 500 in response to either bicalutamide or R1881 (lack of duplicates for R1881 sample precluded using statistical significance – the relaxed criteria results in 58 bicalutamide-induced probe sets as opposed to the original list of 48). The results are shown in the graph (right) color coded by the Venn diagram (left). (f) Probe sets with low variability (standard deviation ≤ 100 and/or coefficient of variation $\leq 50\%$) were filtered out with the remaining probe sets being used in an unsupervised clustering algorithm to identify related subgroups amongst the nine samples.

Supplementary Fig. 1 – <u>Expression profiling of seven HS/HR xenografts</u> (a) Seven 0.5 cm³ tumors from each xenograft, grown in either intact (HS) or castrated (HR) mice, were pooled and total RNA was extracted to generate a single sample. After processing, the cRNA was hybridized to the Affymetrix U95A chip and the microarray data was analyzed by Microarray Suite. Background elements which did not significantly vary (standard deviation < 1000 and coefficient of variation < 1) or were not detected in any of the samples (defined as perfect match hybridization not significantly different than mismatch control signal intensity) were filtered out. The remaining elements, representing 1,056 genes, were then used by an unsupervised learning algorithm to generate a hierarchical clustering diagram. (b) The microarray data, obtained as described in a, was reanalyzed using Microarray Suite whereby each pair was condensed into a single dataset that represents the ratio in expression between each HS xenograft and its HR counterpart. In addition, using a number of parameters as defined by Microarray Suite software such as fold-change, absolute signal intensity and the confidence in each probe set given by perfect match/mismatch ratios, elements from the

condensed dataset were assigned an independent designation of increase (I), marginal increase (MI), no change (NC), marginal decrease (MD) or decrease (D). Elements assessed as NC in all of the xenograft pairs were filtered out to generate a list of 3,774 genes that was then used by an unsupervised learning algorithm to generate a hierarchical

5   clustering diagram. **(c)** Each of the designations described in **b** was assigned a value ranging from +1 (I) to -1 (D) and a score for each element was produced by summing the values across each of the seven HS/HR xenograft pairs. Elements with the highest absolute scores are shown.

Supplementary Fig. 2 – 48 bicalutamide-induced probe sets in LNCaP-AR
10   cells.

Supplementary Fig. 3 – Model of prostate cancer progression   Hormone therapy, consisting of androgen-lowering drugs and competitive AR antagonists, decreases the number of active receptors leading to a clinical response (HS disease). Failure of therapy (HR disease) results from increased receptor level which inverts the

15   response to antagonists and amplifies the response to all ligands – residual androgens, antagonists and other steroids.

Detailed Description of the Invention

Unless otherwise defined, all terms of art, notations and other scientific terms or
20   terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the

25   art. Many of the techniques and procedures described or referenced herein are well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies described in see Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995) and Sambrook et al., Molecular Cloning: A Laboratory

30   Manual 2nd. edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer defined protocols

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and/or parameters unless otherwise noted.

As used herein, the term "polynucleotide" means a polymeric form of nucleotides of at least about 10 bases or base pairs in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide, and is meant to include single and double stranded forms of DNA.

As used herein, the term "polypeptide" means a polymer of at least about 6 amino acids. Throughout the specification, standard three letter or single letter designations for amino acids are used.

The terms "agonist" and "agonistic" when used herein refer to a molecule which is capable of, directly or indirectly, substantially inducing, promoting or enhancing biological activity or activation of a molecule such as AR. The terms "antagonist" and "antagonistic" when used herein refer to a molecule which is capable of, directly or indirectly, substantially inhibiting biological activity or activation of a molecule such as AR.

"Treatment" or "therapy" refer to both therapeutic treatment and prophylactic or preventative measures.

The term "therapeutically effective amount" refers to an amount of a drug effective to treat a disease or disorder in a mammal. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (*i.e.*, slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the disorder. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy *in vivo* can, for example, be measured by assessing tumor burden or volume, the time to disease progression (TTP) and/or determining the response rates (RR).

"Mammal" for purposes of treatment or therapy refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

The terms "cancer", "cancerous", or "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma,

blastoma, sarcoma, and leukemia. More particular examples of such cancers include breast cancer, ovarian cancer, colon cancer, colorectal cancer, rectal cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, Hodgkin's and non-Hodgkin's lymphoma, testicular cancer, esophageal cancer, gastrointestinal cancer, renal cancer, pancreatic cancer, glioblastoma, cervical cancer, glioma, liver cancer, bladder cancer, hepatoma, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

A. Brief Characterization of Features of the Invention

The invention is based on the discoveries disclosed herein that a modest (3-5 fold) increase in androgen receptor (AR) mRNA in prostate cancer cells was the only expression change consistently associated with developing resistance to antiandrogen therapy. This increase in AR mRNA and protein was both necessary and sufficient to convert prostate cancer growth from a hormone sensitive to hormone refractory stage, and was dependent on a functional ligand-binding domain. Furthermore, AR antagonists displayed agonist activity in cells with increased AR levels, and this antagonist/agonist conversion was associated with alterations in the pattern of coactivators and corepressors recruited to the promoter of AR target genes. Increased levels of AR confer resistance to anti-androgens by amplifying signal output from low levels of residual ligand and altering the normal response to antagonists. The findings provide insight toward the design of novel antiandrogens.

One embodiment of the invention disclosed herein is a method of testing compounds for an effect on a mammalian prostate cancer cell comprising contacting the compound to be tested with a the mammalian prostate cancer cell, wherein the mammalian cancer cell expresses an exogenous wild type androgen receptor (AR) polynucleotide that encodes the AR polypeptide of SEQ ID NO:1 such that the levels of mRNA in the cell that encode the AR polypeptide or the AR polypeptide are at least about 2 fold higher than normal/endogenous AR mRNA levels in a mammalian prostate cell; and then comparing one or more characteristics of the mammalian prostate cancer cell to which the compound was administered with the same one or more characteristics of a control mammalian prostate cancer cell to which the compound has not been

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administered, wherein a difference in one or more of the one or more characteristics indicates that the compound has an effect on the mammalian prostate cancer cell.

A test compound which binds AR may then be further screened for the inhibition of a specific physiological activity (e.g. tyrosine kinase activity). Such an embodiment includes, for example determining whether said test compound inhibits the signaling of AR by utilizing molecular biological protocols to create recombinant contracts whose enzymological and biological properties can be examined directly. Enzymology is performed for example, by measuring tyrosine kinase activity in vitro or in ARS expressing cells using standard assays.

Certain discoveries and physiological processes associated with the invention are discussed below.

Expression profiling links increased AR levels to HR progression

To examine the range of HR mechanisms in a relatively unbiased manner, we performed global gene expression profiling on seven "isogenic" hormone sensitive (HS) and HR human prostate cancer xenograft pairs (14 total xenografts). All HR sublines were derived directly from their HS parental lines by passage in castrate mice and compared with HS tumors with similar passage numbers in intact mice[23-26]. The microarray dataset was analyzed using a number of bioinformatic strategies, including unsupervised and supervised learning. First, we asked if any subgroups of HR prostate cancer could be identified using an unsupervised hierarchical clustering algorithm. Each HR xenograft clustered with its HS counterpart, consistent with the fact that these pairs are isogenic (**Supplementary Fig. 1a**). Since xenograft-specific expression signatures are likely to obscure gene expression changes responsible for the HS-to-HR transition[27], we condensed each HS/HR pair into a single dataset representing fold-change in expression. Again, no closely related subgroups emerged (**Supplementary Fig. 1b**), suggesting either that different mechanisms were responsible for the HS-to-HR transition in each xenograft or that any common mechanism involved too few genes to influence the clustering pattern. We explored the latter possibility using an algorithm to identify any elements that consistently changed during the HS-to-HR transition (**Supplementary Fig. 1c**). Remarkably, out of 12,559 probe sets, only one – directed against the *AR* cDNA – was differentially expressed in all seven HS/HR pairs (**Fig. 1,**

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top). Notably, the second highest ranked probe set, upregulated in five of seven pairs, was also directed against *AR*. Consistent with the RNA data, immunoblots showed higher levels of AR protein in HR tumors than their parental HS counterparts (**Fig. 1**, bottom). The fold-change in AR protein (based on analysis of individual xenograft tumors) did not perfectly correlate with the fold-change in *AR* mRNA (based on expression analysis of pools of tumors for each xenograft), possibly due to sample-to-sample variation. Alternatively, post-transcriptional mechanisms may affect steady state AR protein levels[28]. Nonetheless, these data raise the possibility of a common final mechanism of resistance to hormone therapy.

Increased AR levels are sufficient for HR progression

To determine if increased AR protein concentration plays a causal role in the HS-to-HR transition, we introduced an epitope-tagged wildtype *AR* cDNA by retrovirus infection into HS LNCaP human prostate cancer cells. The three-fold increase in AR levels in LNCaP-AR cells (**Fig. 2a**) mimics the expression difference observed in the LNCaP HS/HR pair studied in the microarray experiment. To test whether increased AR expression causes the HS-to-HR transition, we designed two *in vitro* assays to mimic the clinical circumstances of HR disease. The first measures the ability to grow in low androgen concentrations; the second measures growth in the presence of the antiandrogen bicalutamide. As expected, LNCaP cells infected with the empty vector failed to grow in steroid-depleted, charcoal-stripped serum unless supplemented with 100 pM of the synthetic androgen, R1881. In contrast, LNCaP-AR cells grew in at least 80% lower concentrations of R1881 (**Fig. 2b**). LNCaP-AR cells were also resistant to bicalutamide (**Fig. 2c**). Next, we asked if increased AR expression was sufficient, *in vivo*, to confer resistance to hormone therapy achieved through surgical castration using two xenograft models, LNCaP and LAPC4. HS LAPC4 cells were infected with AR lentivirus, demonstrated to express about three-fold more AR protein than vector-infected controls (**Fig. 2d**, inset), then implanted into the flanks of intact or castrated male SCID mice. AR overexpression shortened the latency of tumor formation by more than 50% in castrated animals in the LAPC4 and LNCaP models (**Fig. 2d,e**).

Increased AR levels are necessary for HR progression

We used stable RNA interference to address the reciprocal question of whether the increase in AR expression observed in HR xenografts was necessary for developing resistance to hormone therapy. Knockdown of AR levels in HR LAPC4 cells was achieved using a lentivirus vector expressing a short hairpin RNA (shRNA) against AR

5   *cis*-linked with a GFP-expression cassette (**Fig. 3a**). After implantation into the flanks of castrated male mice, AR shRNA-infected tumors grew more slowly than vector-infected controls (**Fig. 3b**). Moreover, those tumors that did grow did not express GFP when compared to vector-infected controls (**Fig. 3b**, bottom inset) and still expressed AR protein, indicating selection for cells that escaped AR knockdown. Parallel studies of AR

10 knockdown in HR LNCaP sublines gave similar results (**Fig. 3c**).

HR progression remains ligand-dependent

To determine the mechanism by which increased AR levels cause HR disease, we considered two possibilities: (1) high levels of receptor lead to constitutive activation in

15 the absence of ligand, consistent with a recent study of AR[29] (a ligand-independent model), or (2) high levels of receptor sensitize the cell to the residual amounts of ligand remaining after testosterone lowering therapy (a ligand-dependent mass action model). To distinguish between these models, we introduced two AR mutations, N705S and R752Q, independently into the LBD to selectively impair ligand-binding without

20 interfering with ligand-independent functions. N705S is associated with complete androgen insensitivity syndrome, whereas R752Q is found in patients with partial androgen insensitivity syndrome[30]. As expected, both mutations impaired ligand-binding in a radiolabeled R1881-binding assay (**Fig. 4a**). Consistent with disease severity, R752Q retained low levels of ligand-binding and transcriptional activity in a reporter assay, and

25 serves as an internal control to ensure proper folding of at least one LBD mutant. Overexpression of either LBD mutant AR construct in LNCaP cells, even at levels approximately 10-fold higher than endogenous AR (**Fig. 4b**, inset), failed to promote HR growth at levels beyond the vector control in low androgen media, in the presence of bicalutamide (**Fig. 4b**) or in surgically castrated mice (**Fig. 4c**). These data establish

30 that AR must bind ligand to confer HR growth and imply that a modest increase in receptor concentration permits AR to utilize the lower levels of androgens present in castrate patients. This conclusion also suggests that the widely used term "androgen-

independent" may be a misleading description of HR prostate cancer.

Increased AR levels promote HR progression through genotropic mechanisms

The availability of these *in vitro* assays for AR function offered an opportunity to address the additional question of whether AR induces the HS-to-HR transition through genotropic or non-genotropic functions. Deletion of the nuclear localization signal (NLS) blocked nuclear translocation (**Fig. 4d**) and abolished HR growth in low concentrations of androgen or in the presence of bicalutamide (**Fig. 4e**). Similar results were obtained using a second AR mutant V581F that lacks DNA binding activity[31]. Finally, deletion of the polyproline region of AR reported to bind the SH3 domain of Src (ΔP AR)[32] had no inhibitory effect on AR function in these assays (**Fig. 4e**). Therefore, the previously reported non-genotropic effects of AR on bone growth and prostate cancer cell survival[32-34] are unlikely to play a role in resistance to antiandrogen therapy.

Elevated AR levels convert antagonists to agonists

The ligand-binding mutagenesis studies provide evidence for a mass action model to explain resistance to androgen-lowering hormone therapy. If this mechanism is the sole cause of resistance, then suprapharmacologic concentrations of bicalutamide should overcome the excess levels of AR and block transcriptional activity. Surprisingly, we observed the opposite phenomenon when we examined the effects of high-dose bicalutamide on prostate specific antigen (PSA) expression in cells expressing excess AR. Bicalutamide showed typical antagonist activity in parental LAPC4 cells, as measured by inhibition of *PSA* mRNA and protein expression, but functioned as an agonist in LAPC4 cells expressing increased levels of AR (**Fig. 5a,b**). The antagonist-to-agonist conversion was not unique to bicalutamide or to the LAPC4 cell line, since similar results were observed using other AR antagonists such as cyproterone acetate (**Fig. 5c**) and flutamide and with LNCaP cells (**Fig. 5d**). In addition, increased AR levels conferred responsiveness to noncanonical ligands such as estrogen (**Fig. 5e**), reminiscent of the effects of the T877A AR LBD mutation in LNCaP cells[8].

To determine if this conversion was peculiar to the *PSA* gene or was true for other androgen-regulated genes, we conducted a microarray experiment. LNCaP cells infected with an AR-expressing retrovirus or the empty-vector control were challenged

with increasing doses of R1881 or 10 μM bicalutamide and then processed for hybridization to the Affymetrix U133A chip (**Fig. 6a**). Bicalutamide induced the expression of 48 probe sets (defined as 2-fold increase, $P < 0.05$, 500 minimum expression) in LNCaP-AR cells, whereas no elements were significantly upregulated in control cells (**Fig. 6b,c**). The list was comprised mostly of androgen-regulated genes (**Fig. 6d, Supplementary Fig 2**) including the well known members, *PSA* and kallikrein 2 (*KLK2*)[35]. However, bicalutamide induced only a subset (<10%) of the total number of androgen-regulated genes (> 600 probe sets) (**Fig. 6e**, left). Upon closer examination, the bicalutamide-induced genes (in red) overlapped, for the most part, with the most highly induced, androgen-responsive genes (**Fig. 6e**, right). Consistent with this, unsupervised hierarchical clustering of the samples grouped the profile of genes induced by 10 μM bicalutamide most closely with those induced by low doses (10-30 pM) of R1881 (**Fig. 6f**). Together, these data suggest that increased AR expression globally converts antagonists to weak agonists, and therefore leads to the induction of only the most androgen-sensitive genes.

Antagonist/agonist conversion is associated with altered patterns of coactivator assembly

To address the mechanism by which bicalutamide gains agonist properties in the setting of increased AR levels, we performed chromatin immunoprecipitation experiments to define the components of the AR transcription complex on the promoters of two genes, *PSA* and *KLK2*, activated by bicalutamide in the microarray experiment. After exposure to the classic agonists R1881 (R) or dihydrotestosterone (D), AR and polymerase II were recruited to both promoters regardless of AR level (**Fig. 5f**), as expected from prior work[21,36]. AR was also recruited to both templates after exposure to bicalutamide in vector and AR-overexpressing cells, but polymerase II was present only in the setting of excess AR. However, in AR-overexpressing cells, the repertoire of coactivators recruited to these promoters by bicalutamide was more limited (e.g. SRC1 and not AIB1) when compared with R1881 or dihydrotestosterone. We also examined the effect of increased AR levels on promoter occupancy by corepressors. As expected, NCoR was recruited to both promoters in parental cells following bicalutamide treatment without evidence of histone acetylation or polymerase II

recruitment. However, NCoR recruitment was reduced (*PSA* promoter) or absent (*KLK2* promoter) after bicalutamide treatment in cells with increased AR protein levels. Therefore, a modest change in the level of AR protein can shift the relative abundance of coactivators or corepressors assembled on the promoters of AR target genes, with resultant effects on transcriptional activity.

Discussion

The surprising result from our expression profiling was the universal upregulation of *AR* mRNA in all the HR xenograft models, a finding likely to have clinical relevance based on surveys of AR levels in patient material[7,37]. But can this simple model for HR progression be reconciled with prior work implicating other mechanisms? *AR* gene amplification could clearly result in increased AR levels, but this occurs in a minority of patients and cannot be invoked as the explanation for our xenograft findings, since there was no increase in *AR* copy number during the HS-to-HR transition[38]. Studies of *AR* gene regulation have implicated AR itself as a positive acting transcription factor that binds the *AR* gene and leads to increased *AR* mRNA levels[39]. Therefore, other mechanisms postulated to give increased AR activity, such as increased kinase pathway signaling (ErbB2, Ras, MAPK) or altered coactivator/corepressor ratios, may also lead to increased *AR* mRNA levels, albeit indirectly. Thus, any one of a number of primary molecular events that alter AR activity could cause an increase in *AR* mRNA, suggesting a final common pathway for escape from standard hormone therapy.

One caveat is that our conclusions about AR are based on studies of HS xenograft models, nearly all of which have been derived from men with HR disease. The question of how HS growth is "restored" when such tumors are explanted into intact male mice is a long-debated paradox in the prostate cancer field, and the mechanism remains unknown. We previously provided evidence that such explants contain a mixture of HS and HR clones, and that HR sublines develop through clonal expansion under the selective pressure of androgen deprivation[40]. The HS clones that evolve from such explants may be HS only in relative terms, reflecting a transition state in the continuum between truly hormone-naive prostate cancer (no prior exposure to antiandrogen therapy) and full blown HR disease. It remains to be determined whether

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AR upregulation is sufficient to confer HR growth to hormone-naïve cells. Newer transgenic or knockout models of murine prostate cancer may provide an opportunity to address this question in a "cleaner" experimental system[41,42]

The simplest model to explain how increased expression of AR can confer resistance to anti-androgen therapy is mass action. According to this model, the 3-5 fold increase in receptor levels observed in our xenograft models can compensate for low ligand levels and restore AR signaling. However, the fact that increased receptor levels cause antagonists to function as agonists suggests another level of complexity. Our comparison of genes induced by bicalutamide to those induced by a range of androgen doses indicates that antagonists function as weak agonists in the setting of increased AR levels. Comparative analysis of the transcription complex assembled on the promoters of AR target genes revealed a potential mechanism. Specifically, a more limited repertoire of coactivators is recruited to AR target genes after stimulation with bicalutamide, suggesting that suboptimal ligands cannot assemble the optimal array of cofactors for maximal transcriptional activity (**Supplementary Fig. 3**). Because steroid receptor-antagonist complexes adopt a variety of conformations[43,44], antagonist-bound AR may be unable to bind the full complement of coactivator machinery.

The molecular basis for loss of antagonism is less apparent. Previous work has demonstrated that the agonist versus antagonist response of nuclear receptors like ER is altered by increased expression of coactivators (increased SRC1) or by decreased expression of corepressors (decreased NCoR)[45,46]. Our data establish that increased expression of the nuclear receptor itself causes a similar outcome, perhaps by upsetting the balance of corepression and coactivation in the cell. Additional experiments examining each of these components are required to sort through these or alternative explanations.

The clinical relevance of antagonist/agonist conversion should also be considered. About 30% of men whose disease progresses during treatment with AR antagonists experience a paradoxical fall in serum PSA levels when the antagonist is discontinued, called anti-androgen withdrawal syndrome[47]. One proposed mechanism is mutation in the *AR* gene, based on the fact that flutamide functions as an agonist in cells expressing the T877A AR mutation[8]. Although compelling, this mechanism cannot account for all cases because recent estimates of the frequency of AR mutations in HR

patients are too low[5]. Our findings suggest that patients with antiandrogen withdrawal syndrome may be those with the highest level of AR upregulation.

Perhaps the most important implication of this work is toward the development of novel antiandrogens. The fact that an intact LBD is required for AR to cause resistance to hormone therapy provides compelling rationale for the design of novel antagonists that exploit existing knowledge of this well defined binding pocket[48]. Because AR action appears to be mediated exclusively through genotropic mechanisms, one can also envision drugs that prevent AR nuclear translocation or impair assembly of AR transcription complexes on target genes. Finally, it will be important to determine if the mechanisms of antiandrogen resistance implicated here have relevance for other hormone-dependent diseases such as breast cancer.

Illustrative Methods Useful with the Invention

Selection and Transformation of Host Cells

Host cells such as prostate cancer cells can transfected or transformed with expression or cloning vectors described herein for the expression of the human AR proteins and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991) and Sambrook et al., supra.

A wide variety of methods of transducing mammalian cells are known in the art, for example, using reagents and methods such as viral vectors (e.g. the retroviral vectors disclosed in the Examples below), lipids (e.g. lipofection), $CaPO_4$ and electroporation etc. Depending on the host cell used, transformation is performed using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van

der Eb, Virology, 52:456-457 (1978) can be employed. General aspects of mammalian cell host system transformations have been described in U.S. Patent No. 4,399,216. However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

Suitable host cells for cloning or expressing the AR DNA in the vectors herein include various prostate cancer cell lines such as LNCaP lines, DU145 and TsuPr1, other transfectable or transducible prostate cancer cell lines, primary cells (PrEC), as well as a number of mammalian cells routinely used for the expression of recombinant proteins (e.g., COS, CHO, 293, 293T cells).

Selection and Use of a Replicable Vector

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding AR may be inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan.

The AR proteins may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide (e.g. the FLAG tag disclosed herein), which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the AR DNA that is inserted into the vector. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin

II leaders. For yeast secretion the signal sequence may be, *e.g.*, the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders, the latter described in U.S. Patent No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression, mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders.

Both expression and cloning vectors typically contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. A wide range of host-vector systems suitable for the expression of AR are available, see for example, Sambrook et al., 1989, supra; Current Protocols in Molecular Biology, 1995, supra). Preferred vectors for mammalian expression include but are not limited to pcDNA 3.1 myc-His-tag (Invitrogen) and the retroviral vector pSRαtkneo (Muller et al., 1991, MCB 11:1785). Using these expression vectors, AR can be expressed in prostate cancer and non-prostate cell lines, including for example LNCaP, 293, 293T, rat-1, NIH 3T3 and TsuPr1. The host-vector systems of the invention are useful for the production of a AR protein or fragment thereof. Such host-vector systems can be employed to study the functional properties of AR and AR mutations or analogs.

Expression and cloning vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the AR nucleic acid, such as DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as

described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid YRp7 (Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)). The trp1 gene provides a selection marker for a

5   mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, Genetics, 85:12 (1977)).

        Throughout this application, various publications are referenced (within parentheses for example). The disclosures of these publications are hereby incorporated

10   by reference herein in their entireties. In order to facilitate an understanding of various typical aspects of the invention, certain aspects of these incorporated materials are reproduced herein.

        The present invention is not to be limited in scope by the embodiments disclosed herein, which are intended as single illustrations of individual aspects of the

15   invention, and any that are functionally equivalent are within the scope of the invention. Various modifications to the models and methods of the invention, in addition to those described herein, will become apparent to those skilled in the art from the foregoing description and teachings, and are similarly intended to fall within the scope of the invention. Such modifications or other embodiments can be practiced without departing

20   from the true scope and spirit of the invention. However, the invention is only limited by the scope of the appended claims.

EXAMPLES

25   Example 1: Typical Materials

        Oligonucleotide U95A and U133A gene arrays were purchased from Affymetrix. Charcoal-stripped dextran-treated fetal bovine serum was obtained from Omega Scientific. Bicalutamide was obtained from the UCLA Investigational Drug Pharmacy and dissolved in acetone. Cold and ^{3}H-R1881 were obtained from NEN Life Sciences.

30   AR antibody N-20 (Santa Cruz) and Flag antibody M2 (Sigma) were used in the immunoblot assays. Secreted PSA was measured by ELISA (American Qualex). Protein extracts were prepared in high detergent buffer (2% SDS) to ensure total cell lysis.

Example 2: Typical DNA Constructs and PCR primers

pCSUACG (U6-shRNAαAR;CMV-GFP) was constructed by ligating the *Bam*HI/*Eco*RI digests of pCSCG and the U6-shRNAαAR PCR product. The U6-shRNAαAR PCR was performed using a hU6-containing plasmid at a 60°C annealing temperature with primers: 5'GGGGGATCCCAAGGTCGGGCAGGAAGAGGGCCTATTTCC-3' and 5'-GGGGAATTCAAAAAGCACTGCTACTCTTCAGCATCTCTTGAATGCTGAAGAGTAGCAGTGCGGTGTTTCGTCCTTTCCACAAGATATATAA-3'. pCSCA (CMV-AR) was created by subcloning the *Xba*I fragment of pSRα-AR into the *Nhe*I site of pCSCG. *AR* mutants were made by standard PCR-based site-directed mutagenesis using the QuikChange Kit (Stratagene). ΔNLS contains three point mutations (K618M, K632M, K633M) previously shown to disrupt nuclear import[49]. ΔPro contains a deletion of amino acids 372-381, based on prior work[32]. ARR₂Pb-Luciferase was kindly provided by Robert Matusik (Vanderbilt). *PSA* RT-PCR performed with primers: 5'-GGTGACCAAGTTCATG-CTGTG-3' and 5'GTGTCCTTGATCCACTTCCG-3'.

Example 3: Typical *In vitro* and *in vivo* growth experiments

LNCaP (ATCC) and LAPC4 cells were maintained in Iscove's medium supplemented with 10% fetal bovine serum. LNCaP-AR and LNCaP-vector were derived from infection with the pSRα-AR or pSRα retrovirus, respectively, and selection in 500 ng/ml of G418. LNCaP-AR, LNCaP-vector, LAPC4-AR, and LAPC4-vector in other experiments were derived by infection with the pCSCA or pCSC lentivirus, respectively, without selection (>90% infection). For *in vitro* experiments, LNCaP or LAPC4 cells stably infected with different constructs were androgen-starved by growth in charcoal-stripped serum for 3-5 d. 5x10⁴ cells were plated per well in media containing 10% charcoal-stripped serum supplemented with various concentration of R1881 or in media containing 10% full serum with various concentration of bicalutamide. Colonies were visualized with crystal violet staining 2 weeks later. *In vivo* tumorigenicity was measured by injection of 5 x 10⁵ LAPC4 or 1 x 10⁶ LNCaP cells in 100 μl of Matrigel (Collaborative Biomedical) subcutaneously into the flanks of intact or

castrated male SCID mice. Tumor size was measured weekly in three dimensions using caliber as described[26]. AR knockdown was performed by infection of HR LAPC4 with shRNA AR lentivirus. Tumors which grew in castrated mice were explanted, and analyzed by flow cytometry for the percentage of GFP-positive cells. All mouse experiments were performed in compliance with the guidelines of the Animal Research Committee (ARC) of the UCLA.

Example 4: Typical Microarray experiments and data analysis

HS and HR xenograft pairs for the microarray study were collected from three institutions. LUCaP23, 35, and 41 were developed at University of Washington; CWR22 was developed by Case Western Reserve University and kindly provided by University of North Carolina at Chapel Hill; LAPC4 and 9 were developed at the University of California at Los Angeles; LNCaP was purchased from ATCC and implanted into mice. The HS xenografts were either grown in intact nude mice or SCID male mice, and their HR counterparts were developed by serial passage in castrated male mice. Microarray experiments were performed and data analyzed according to manufacture's instructions (Affymetrix). We extracted total RNA from a pool of two to eight tumors with comparable sizes and serum PSA levels for each xenograft using TriReagent (Molecular Research Center) and RNeasy (Qiagen). For each sample, 15 μg of total RNA was used to generate double stranded cDNA and the cRNA was transcribed with biotin-labeled nucleotides (ENZO Diagnostics). The cRNA was fragmented and hybridized to U95A microarray (Affymetrix). Scanned images were used for absolute and comparison analysis (Affymetrix manual). The microarray data in **Fig. 6** were generated through the Genespring program (Silicon Genetics).

Example 5: Typical Chromatin immunoprecipitation

LNCaP-AR or LNCaP-vector were androgen-starved and challenged with either vehicle, 100 pM of R1881 (R), 1 nM of DHT (D), or 10 μM of bicalutamide (B) for 1 h. Soluble chromatin was prepared after formaldehyde crosslinking and sonication. Specific IgGs against AR, N-CoR, PolII, Ac-H3/4, SRC1, TIF2, AIB1, and PCAF were used to immunoprecipitate protein-bound DNA fragments. After reversing the crosslinking, PCR reactions were performed to amplify the promoter regions of *KLK3/PSA* or

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KLK2[21].

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Table 1: Androgen Receptor Polynucleotide and Polypeptide Sequences

NM_000044 gi:21322251)
See, e.g. Chang et al., Science 240 (4850), 324-326 (1988)

AR Polynucleotide sequence

CGAGATCCCGGGGAGCCAGCTTGCTGGGAGAGCGGGACGGTCCGGAGCAAGCCCAGAGGCAGAGGAGG
CGACAGAGGGAAAAAGGGCCGAGCTAGCCGCTCCAGTGCTGTACAGGAGCCGAAGGGACGCCACCACGC
CAGCCCCAGCCCGGCTCCAGCGACAGCCAACGCCTCTTGCAGCGCGGCGGCTTCGAAGCCGCCGCCCG
GAGCTGCCCTTTCCTCTTCGGTGAAGTTTTTAAAAGCTGCTAAAGACTCGGAGGAAGCAAGGAAAGTG
CCTGGTAGGACTGACGGCTGCCTTTGTCCTCCTCCTCTCCACCCCGCCTCCCCCCACCCTGCCTTCCC
CCCCTCCCCCGTCTTCTCTCCCGCAGCTGCCTCAGTCGGCTACTCTCAGCCAACCCCCCTCACCACCC
TTCTCCCCACCCGCCCCCCCGCCCCCGTCGGCCCAGCGCTGCCAGCCCGAGTTTGCAGAGAGGTAACT
CCCTTTGGCTGCGAGCGGGCGAGCTAGCTGCACATTGCAAAGAAGGCTCTTAGGAGCCAGGCGACTGG
GGAGCGGCTTCAGCACTGCAGCCACGACCCGCCTGGTTAGGCTGCACGGTGGGAGAGAACCCTCTGTTTT
CCCCCACTCTCTCTCCACCTCCTCCTGCCTTCCCCACCCCGAGTGCGGAGCCAGAGATCAAAAGATGA
AAAGGCAGTCAGGTCTTCAGTAGCCAAAAAACAAAACAAAACAAAAACAAAAAAGCCGAAATAAAAGAA
AAAGATAATAACTCAGTTCTTATTTGCACCTACTTCAGTGGACACTGAATTGGAAGGTGGAGGATTT
TGTTTTTTTCTTTTAAGATCTGGGCATCTTTTGAATCTACCCTTCAAGTATTAAGAGACAGACTGTGA
GCCTAGCAGGGCAGATCTTGTCCACCGTGTGTCTTCTTCTGCACGAGACTTTGAGGCTGTCAGAGCGC
TTTTTGCGTGGTTGCTCCCGCAAGTTTCCTTCTCTGGAGCTTCCCGCAGGTGGGCAGCTAGCTGCAGC
GACTACCGCATCATCACAGCCTGTTGAACTCTTCTGAGCAAGAGAAGGGGAGGCGGGGTAAGGGAAGT
AGGTGGAAGATTCAGCCAAGCTCAAGGATGGAAGTGCAGTTAGGGCTGGGAAGGGTCTACCCTCGGCC
GCCGTCCAAGACCTACCGAGGAGCTTTCCAGAATCTGTTCCAGAGCGTGCGCGAAGTGATCCAGAACC
CGGGCCCCAGGCACCCAGAGGCCGCGAGCGCAGCACCTCCCGGCGCCAGTTTGCTGCTGCTGCAGCAG
CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAAGAGAC
TAGCCCCAGGCAGCAGCAGCAGCAGCAGCAGGGTGAGGATGGTTCTCCCCAAGCCCATCGTAGAGGCCCCA
CAGGCTACCTGGTCCTGGATGAGGAACAGCAACCTTCACAGCCGCAGTCGGCCCTGGAGTGCCACCCC
GAGAGAGGTTGCGTCCCAGAGCCTGGAGCCGCCGTGGCCGCCAGCAAGGGGCTGCCGCAGCAGCTGCC
AGCACCTCCGGACGAGGATGACTCAGCTGCCCCATCCACGTTGTCCCTGCCTGGGCCCCCACTTTCCCCG
GCTTAAGCAGCTGCTCCGCTGACCTTAAAGACATCCTGAGCGAGGCCAGCACCATGCAACTCCTTCAG
CAACAGCAGCAGGAAGCAGTATCCGAAGGCAGCAGCAGCGGGAGAGCGAGGGAGGCCTCGGGGGGCTCC
CACTTCCTCCAAGGACAATTACTTAGGGGGCACTTCGACCATTTCTGACAACGCCAAGGAGTTGTGTA
AGGCAGTGTCGGTGTCCATGGGCC

AR Polypeptide sequence

MEVQLGLGRVYPRPPSKTYRGAFQNLFQSVREVIQNPGPRHPEAASAAPPGASLLLLQQQQQQQQQQQ
QQQQQQQQQQQETSPRQQQQQQGEDGSPQAHRRGPTGYLVLDEEQQPSQPQSALECHPERGCVPEPG
AAVAASKGLPQQLPAPPDEDDSAAPSTLSLLGPTFPGLSSCSADLKDILSEASTMQLLQQQQQEAVSE
GSSSGRAREASGAPTSSKDNYLGGTSTISDNAKELCKAVSVSMGLGVEALEHLSPGEQLRGDCMYAPL
LGVPPAVRPTPCAPLAECKGSLLDDSAGKSTEDTAEYSPFKGGYTKGLEGESLGCSGSAAAGSSGTLE
LPSTLSLYKSGALDEAAAYQSRDYYNFPLALAGPPPPPPPPPHPHARIKLENPLDYGSAWAAAAAQCRY
GDLASLHGAGAAGPGSGSPSAAASSSWHTLFTAEEGQLYGPCGGGGGGGGGGGGGGGGGGGGGGGEAG
AVAPYGYTRPPQGLAGQESDFTAPDVWYPGGMVSRVPYPSPTCVKSEMGPWMDSYSGPYGDMRLETAR
DHVLPIDYYFPPQKTCLICGDEASGCHYGALTCGSCKVFFKRAAEGKQKYLCASRNDCTIDKFRRKNC
PSCRLRKCYEAGMTLGARKLKKLGNLKLQEEGEASSTTSPTEETTQKLTVSHIEGYECQPIFLNVLEA
IEPGVVCAGHDNNQPDSFAALLSSLNELGERQLVHVVKWAKALPGFRNLHVDDQMAVIQYSWMGLMVF
AMGWRSFTNVNSRMLYFAPDLVFNEYRMHKSRMYSQCVRMRHLSQEFGWLQITPQEFLCMKALLLFSI
IPVDGLKNQKFFDELRMNYIKELDRIIACKRKNPTSCSRRFYQLTKLLDSVQPIARELHQFTFDLLIK
SHMVSVDFPEMMAEIISVQVPKILSGKVKPIYFHTQ

WHAT IS CLAIMED IS:

1.   A method of examining the physiological effect of a compound on a mammalian prostate cancer cell, the method comprising:

    (a) contacting the compound to be tested with a mammalian prostate cancer cell, wherein the mammalian prostate cancer cell expresses:

        (i) an exogenous wild type polynucleotide that encodes the AR polypeptide of SEQ ID NO:1;

        (ii) a polynucleotide that encodes the AR polypeptide of SEQ ID NO:1 having a deletion, insertion or substitution of at least one amino acid in the AR polypeptide of SEQ ID NO:1,

    such that the total levels of mRNA in the cell that encode the AR polypeptide or the total levels of AR polypeptide are at least 2 fold higher than normal/endogenous AR mRNA or AR polypeptide in the cell; and

    (b) examining one or more physiological characteristics of the mammalian prostate cancer cell to which the compound is administered, so that the physiological effect of the compound on the mammalian prostate cancer cell is examined.

2.   The method of claim 1, further comprising examining the physiological effect of a plurality of compounds on the mammalian prostate cancer cell, wherein an observable difference in one or more physiological characteristics exerted by a first compound as compared to one or more physiological characteristics exerted by a second compound indicates that the first compound has a stronger or weaker physiological effect than the second compound on the mammalian prostate cancer cell.

3.   The method of claim 1, wherein the method of performed in a high throughput format.

4.   The method of claim 1, wherein the method of performed in a low throughput format.

5.   A method of testing compounds for an effect on a mammalian prostate cancer cell, the method comprising,

(a) contacting the compound to be tested with a the mammalian prostate cancer cell, wherein the mammalian cancer cell expresses an exogenous wild type androgen receptor (AR) polynucleotide that encodes the AR polypeptide of SEQ ID NO:1 such that the levels of mRNA in the cell that

5   encode the AR polypeptide or the AR polypeptide are at least about 2 fold higher than normal/endogenous AR mRNA levels in a mammalian prostate cell;

(b) comparing one or more characteristics of the mammalian prostate cancer cell to which the compound was administered with the same one or more characteristics of a control mammalian prostate cancer cell to which the

10   compound has not been administered,

wherein a difference in one or more of the one or more characteristics indicates that the compound has an effect on the mammalian prostate cancer cell.

6.   A method of testing compounds for an effect on a mammalian cell, the method

15   comprising,

(a) contacting the compound to be tested with the mammalian cell, wherein the mammalian cell expresses an exogenous wild type or mutated protein of interest, such as the estrogen receptor, such that the total levels of mRNA in the cell that encode the protein of interest or the total protein levels of the

20   protein of interest are at least 2 fold higher than normal/endogenous mRNA or polypeptide of the protein of interest, such as the estrogen receptor, in the cell;

(b) comparing one or more characteristics of the mammalian cell of (a) to which the compound is administered with the same one or more characteristics of a

25   control mammalian cell to which the compound has not been administered, wherein a difference in one or more characteristics indicates that the compound has an effect on the mammalian cancer cell or mammalian cell.

7.   The method of claim 6, wherein the mammalian cell is a cancer cell.

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8.   The method of claim 7, wherein the cancer cell is a breast, ovarian or prostate cancer cell.

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9. The method of claim 6, further comprising examining the physiological effect of a plurality of compounds on the mammalian cell, wherein an observable difference in one or more physiological characteristics exerted by a first compound as compared to one or more physiological characteristics exerted by a second compound indicates that the first compound has a stronger or weaker physiological effect than the second compound on the mammalian cell.

10. A method of treating a hormone refractory prostate cancer in a patient, the method comprising administering to the patient an agent that decreases or affects the biological function of the androgen receptor by affecting the androgen receptor ligand-binding, nuclear translocation, or by affecting DNA-binding, or through altering formation of coactivator or corepressor complexes associated with the androgen receptor.

11. A method of treating a hormone refractory prostate cancer in a patient, the method comprising administering to the patient and an agent that decreases or affects the biological function of the androgen receptor through affecting the androgen receptor DNA levels, androgen mRNA levels, or androgen protein levels.

12. The method of claim 11, wherein the androgen receptor protein level is decreased through modulation of signal transduction pathways such as targeting EGF receptors that crosstalk to the androgen receptor.

13. The method of claim 11, wherein the androgen receptor protein level is decreased by the induction of cellular degradation pathways such as proteosome degradation machinery.

14. The method of claim 11, wherein the androgen receptor protein level is decreased by dissociating the androgen receptor from heat shock proteins that maintain the androgen receptor integrity.

15. The method of claim 11, wherein the androgen receptor protein level is

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decreased using androgen receptor antisense or mRNA knockdown technology.

16.   A method of treating a hormone refractory prostate cancer in a patient, the method comprising administering to the patient and an agent that decreases or affects the biological function of the androgen receptor through modifying the androgen receptor protein.

17.   The method of claim 16, wherein the androgen receptor protein is modified by modifying the polynucleotide or polypeptide sequence of the androgen receptor or by posttranslational modifications of the androgen receptor including but not restricted to phosphorylation, acetylation, ubiquitination, and sumolation.

18.   A method of treating a disease or condition, which is resistant to a drug, or a treatment, or combination of a drug and a treatment by increasing the concentration of the protein that is the target of the drug or treatment, the method comprising administering one or more agents or utilizing a technique that affects the biological function of the protein through means described in claims 9, 10 or 15.

19.   The method of claim 18, wherein the disease or condition is hormone refractory prostate cancer, in which the androgen receptor DNA, mRNA, or protein levels is increased in prostate cancer cells after surgical or medical castration, or treatments with anti-androgen therapy, or the combination of castration and anti-androgen therapy.

20.   The method of claim 18, wherein the disease or condition is hormone refractory breast cancer, in which the estrogen receptor DNA, mRNA, or protein levels is increased in breast cancer cells after hormone therapy such as treatments with tamoxifen or raloxifene.

METHODS AND MATERIALS FOR ASSESSING PROSTATE CANCER THERAPIES

Abstract of the Disclosure

5   Using microarray-based profiling of isogenic prostate cancer xenograft models, we found that a modest (3-5 fold) increase in androgen receptor (AR) mRNA was the only expression change consistently associated with developing resistance to antiandrogen therapy. Increased levels of AR confer resistance to anti-androgens by amplifying signal output from low levels of residual ligand and altering the normal

10   response to antagonists. This disclosure provides cell based assays for use in the examination of new therapeutic modalities and provides insight toward the design of novel antiandrogens.

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Figure 1

Figure 2

Figure 3

Figure 4

a

Specific activity vs R1881 (nM)

WT, R752Q, N705S

b

Low-androgen sensitization assay
Antagonist resistance assay

Relative colonies

Vector, WT, N705S, R752Q

AR
β-actin

V, WT, N705S, R752Q

c

Tumor volume (mm³) vs Time (d)

WT, Vector, R752Q, N705S

d

WT, ?NLS

e

Low-androgen sensitization assay
Antagonist resistance assay

Relative colonies

Vector, WT, ?Pro, V581F, ?NLS

AR
β-actin

V, WT, ?Pro, V581F, ?NLS

Figure 5

a

Bicalutamide (μM): GFP 0 0.03 0.3 3.0 30 AR 0 0.03 0.3 3.0 30

PSA (32 cycles)

PSA (28 cycles)

β-actin

b

PSA (ng/mL)

Bicalutamide (μM)

0.1   1   10   100

AR

Vector

c

Cyproterone acetate (nM): GFP 0 16 AR 0 16

PSA

β-actin

d

Bicalutamide (μM): Neo 0 10 AR 0 10

PSA

β-actin

e

PSA (ng/mL)

17β-estradiol (nM)

0.01   0.1   1   10

AR

Vector

Figure 6

a

LNCaP infected with AR-expressing virus or Neo control → 48 h treatment with vehicle, 10 μM bicalutamide or 10, 30, 90, 270 or 810 pM R1881 → Affymetrix U133A chip

b

PSA & KLK2

Vehicle (0.1 %)   Bicalutamide (10 μM)

Fold change (48 probe sets): 100, 10, 1, 0.1

c

Fold change (48 probe sets): 100, 10, 1

R1881 (pM): 0, 10, 30, 90, 270, 810

d

Fold change (665 probe sets): 100, 10, 1, 0.1

R1881 (pM): 0.1, 10, 30, 90, 270, 810

810 pM R1881
10 μM bicalutamide

e

Neo10 μM bicalutamide
Neo0.1% vehicle
AR0.1% vehicle
AR10 μM bicalutamide
AR30 pM R1881
AR90 pM R1881
AR90 pM R1881
AR270 pM R1881
AR810 pM R1881

Normalized expression

f

PSA   KLK2

Vector   AR   Vector   AR
V B R D   V B R D   V B R D   V B R D

10 % input
AR
NCoR
Pol II
Ac-H3/4
SRC-1
TIF-2
AIB-1
PCAF

Supplementary Figure 1

a

Genes (1,056)

HS CWR22
HR CWR22
HS LAPC9
HR LAPC9
HS LuCaP23
HR LuCaP23
HR LuCaP41
HS LuCaP41
HS LAPC4
HR LAPC4
HS LNCaP
HR LNCaP
HS LuCaP35
HR LuCaP35

3   1.5   0

Normalized microarray value

Supplementary Figure 1

b

Genes (3,774)

Samples

CWR22
LAPC4
LuCaP23
LuCaP35
LuCaP41
LNCaP
LAPC9

3 ... 0 ... -3
Fold-change
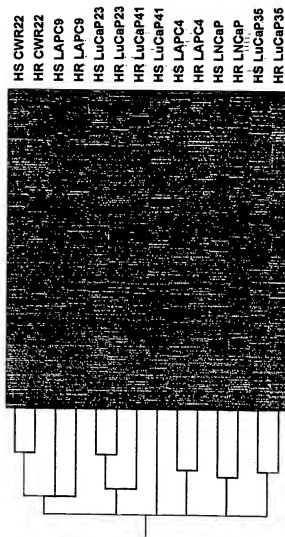
Supplementary Figure 1

c

Gene Name (Acc. No.)	CWR22	LuCaP23	LuCaP35	LuCaP41	LNCaP	LAPC4	LAPC9	Score
Androgen receptor (M23263)	2.5 (I)	3.1 (I)	3.1 (I)	3.6 (I)	2.7 (I)	2.3 (I)	2.2 (I)	7.0
Androgen receptor (M23263)	2.0 (I)	3.1 (I)	2.7 (I)	6.0 (I)	2.0 (I)	1.3 (NC)	1.4 (NC)	5.0
Carbonic anhydrase precursor (AF037335)	1.8 (I)	1.4 (NC)	-1.2 (NC)	4.7 (I)	3.7 (I)	1.1 (NC)	1.5 (I)	4.0
Elongation factor 1 alpha-2 (X70940)	-1.1 (I)	-1.2 (NC)	1.7 (I)	1.1 (I)	1.0 (NC)	1.3 (I)	-1.3 (NC)	4.0
HLA class I locus C heavy chain (X58536)	1.9 (I)	1.1 (NC)	1.1 (NC)	2.5 (I)	1.7 (I)	1.1 (NC)	4.4 (I)	4.0
Platelet-type Phosphofructokinase (D25328)	-3.3 (MD)	-1.8 (D)	-2.7 (D)	-2.4 (D)	-2.2 (D)	1.7 (NC)	1.2 (NC)	-4.5
Secreted cement gland Protein XAG-2 homolog (AF038451)	-10.7 (D)	-1.7 (D)	1.7 (NC)	-2.0 (D)	-2.5 (D)	-3.2 (D)	1.9 (I)	-4.0
Slow MyBP-C (X73114)	-4.1 (D)	-3.8 (D)	-1.5 (NC)	-3.7 (D)	-1.2 (NC)	-1.8 (D)	-1.2 (NC)	-4.0

Supplementary Figure 2

Affy ID	GenBank ID	Gene Name
212005	AL582808	DKFZP566C0424 protein
213017	NM_138340	abhydrolase domain containing 3
201662	D89053.1	Acyl-CoA synthetase 3
216323	XM_054284	alpha-tubulin isotype H2-alpha
211689	AF270487.1	androgen-regulated serine protease (TMPRSS2)
205102	NM_005656	androgen-regulated serine protease (TMPRSS2)
215990	S67779.1	BCL5
203356	NM_014296	calpain 7
222201	AB037736.1	CASP8 associated protein 2
221272	NM_030806.1	chromosome 1 open reading frame 21 (C1ORF21)
222121	NM_015595	DKFZP434D146 protein
212685	NM_015508	DKFZP434J214 protein
209389	M15887.1	endozepine
204580	NM_004117.1	FK506-binding protein 5 (FKBP5)
210892	BC004472.1	general transcription factor II
219312	NM_023929.1	hypothetical protein FLJ12752
219476	NM_024115.1	hypothetical protein MGC4309
210339	BC005196.1	kallikrein 2 (KLK2)
209854	NM_005551	kallikrein 2 (KLK2)
204583	U17040.1	kallikrein 3/prostate specific antigen (PSA/KLK3)
204582	NM_001648	kallikrein 3/prostate specific antigen (PSA/KLK3)
212789	XM_168201	KIAA0056 protein
205862	NM_014868.1	KIAA0575 gene product (KIAA0575)
201551	J03263.1	lysosome-associated membrane glycoprotein (lamp A)

Affy ID	GenBank ID	Gen Name
206205	NM_022782.1	M-phase phosphoprotein 9 (MPHOSPH9)
221965	NM_022782	M-phase phosphoprotein 9 (MPHOSPH9)
208309	NM_006785.1	mucosa associated lymphoid tissue lymphoma translocation gene 1 (MALT1)
214087	NM_002465	myosin binding protein C, slow type
211548	J05594.1	NAD+-dependent 15-hydroxyprostaglandin dehydrogenase (PDGH)
205040	NM_000607.1	orosomucoid 1 (ORM1)
205041	NM_000607.1	orosomucoid 1 (ORM1)
220954	NM_013440.1	paired immunoglobulin-like receptor beta (PILR(BETA))
206178	NM_000929.1	phospholipase A2, group V (PLA2G5)
214443	NM_006505.1	poliovirus receptor (PVR)
218782	NM_014109.1	PRO2000 protein (PRO2000)
205924	BC005035.1	RAB3B
201975	NM_002956.1	restin (Reed-Steinberg cell-expressed intermediate filament-associated protein) (RSN)
201562	NM_003104	sorbitol dehydrogenase (SORD)
216920	M27331.1	T cell receptor gamma-chain
215806	M13231.1	T-cell receptor aberrantly rearranged gamma-chain
209813	M16768.1	T-cell receptor gamma chain VJCI-CII-CIII region
211144	M30894.1	T-cell receptor TI rearranged gamma-chain
201108	NM_003246	thrombospondin 1
222118	AK023869.1	uncharacterized bone marrow protein BM039
219555	NM_018456	uncharacterized bone marrow protein BM039 (BM039)
219551	NM_018456.1	uncharacterized bone marrow protein BM040 (BM040)
209053	BE793789	Wolf-Hirschhorn syndrome candidate 1 /FL=gb:AF083389.1
209309	D90427.1	zinc-alpha2-glycoprotein

Supplementary Figure 2 continued

Supplementary Figure 3

Androgens (ex. Testosterone)
Antiandrogens (ex. Bicalutamide)

HS Prostate Cancer

Hormone Therapy

HR Prostate Cancer